



Faculty of Resource Science and Technology

**CHARACTERIZATION OF ANAEROBIC AMMONIUM OXIDATION
(ANAMMOX) BACTERIA FROM LOCAL WASTEWATER
TREATMENT PLANT**

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**Characterization of Anaerobic Ammonium Oxidation (Anammox) Bacteria from
Local Wastewater Treatment Plant**

Chandrasuriya Lingam

This report is submitted in partial fulfillment of the requirement for
the degree of Bachelor of Science with Honors in

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Department of Molecular Biology

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List of Abbreviations

16S rRNA	16s Ribosomal Ribonucleic Acid	12
16s rDNA	16s Ribosomal Deoxyribonucleic Acid	
Anammox	Anaerobic Ammonium Oxidation	
DNA	Deoxyribonucleic Acid	
dNTPs	Deoxyribonucleic Triphosphate	
NaCl	Sodium Chloride	16
NaCl/CTAB	Sodium Chloride /Cetyl trimethylammonium bromide	
PCR	Polymerase Chain Reaction	18
SDS	Sodium dodecyl sulphate	

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Characterization of Anaerobic Ammonium Oxidation (Anammox) Bacteria from Local Wastewater Treatment Plant

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ABSTRACT

Anaerobic ammonium oxidation (anammox) process plays an important role in biological nitrogen removal. The bacteria catalyzing anammox process belongs to phylum *Planctomycetales*. The objective of this research is to characterize the anammox bacteria from leachate sample collected from a local wastewater treatment plant. Characterization of anammox bacteria includes enrichment, morphology, physico-chemical and molecular analysis. Screening for anammox bacteria was conducted using both enrichment culture and directly from leachate sample. Identification of anammox bacteria from enrichment culture and leachate sample using molecular method involves DNA extraction and PCR. Presence of anammox activity in enrichment culture was detected using physico-chemical method. No bands were obtained in PCR using DNA extracted from enrichment culture. Presence of uncultured *Planctomycete* bacteria with potential anammox activity was detected through molecular analysis directly in leachate sample. Identification of anammox bacteria are advantageous as it can be applied in wastewater treatment plants to the level of ammonium and enhance the quality of discharged effluent.

Keywords: Anammox bacteria, biological nitrogen removal, physico-chemical analysis, molecular analysis, uncultured *Planctomycete* bacteria.

ABSTRAK

Proses pengoksidaan ammonia secara anaerobik memainkan peranan penting di dalam proses penyingkiran nitrogen secara biologi. Bacteria yang memangkinkan proses pengoksidaan ammonia secara anaerobik berasal daripada filum Planctomycetales. Tujuan kajian ini dijalankan adalah untuk mengenal pasti bacteria yang mengoksidakan ammonia secara anaerobik daripada sampel air sisa yang didapati dari pusat rawatan air sisa. Kajian untuk mengenal pasti bacteria anamoks merangkumi pengkulturan bacteria, morfologi, fisiko-kimia dan analisis molekul. Kehadiran bacteria anamokstelah dikaji di dalam kultur and sampel air sisa telah dikenal pasti melalui kaedah molekul menggunakan teknik mengekstrak DNA dan tindak balas rantai polimerasi. Kehadiran aktiviti anamoks di dalam kultur telah dikesan dengan menggunakan teknik fisiko-kimia. Tiada hasil diperolehi menggunakan tindak balas rantai polimerasi menggunakan DNA yang diekstrak daripada kultur. Kehadiran bacteria Planctomycetes yang belum dikultur dengan potensi aktiviti anamoks di dalam sampel air sisa telah dikenal pasti melalui analisis molekul. Kajian bakteria anamoks sangat bermanfaat kerana ia boleh diaplikasikan di dalam pusat rawatan air sisa untuk memperbaiki dan meningkatkan kualiti efluen yang dibuang.

Kata Kunci: Bacteria anamoks, Kitar nitrogen biologi, analisis fisiko-kimia, analisis molekul, bakteria *Planctomycete* yang belum dikultur.

1.0 INTRODUCTION

Biological removal of nitrogen from wastewater involves conventional nitrification and denitrification methods. It is an essential process for biological nitrogen cycle. Anaerobic ammonium oxidation (anammox) process is an alternative nitrogen removal (Mulder *et al*, 1995). Anaerobic ammonium oxidation (anammox) bacteria were responsible for anammox process. It oxidizes ammonium to nitrogen gas with nitrite as electron donor under anaerobic conditions (Van de Graaf *et al*. 1995, 1996; Strous *et al*. 1997). The purpose of this study is to characterize anammox bacteria suspected to be present in leachate sample from local wastewater treatment plant. Leachate sample were collected from anoxic tank containing nitrogenous municipal wastewater from landfill.

Leachate is contaminated water from dumping sites or landfill. It is generated by percolation of precipitation of municipal waste in landfill. The percolating water contaminated with decomposing solid waste material will flow out from landfill sites (Ahel *et al*, 2004). The leachate will then be treated in wastewater treatment plant to remove hazardous chemicals and high level of nutrients. Leachate will undergo a series of treatment consisting of physical, chemical and biological method. Biological leachate treatment method often involves activated sludge process where microorganisms are used to remove nitrogen, phosphorus and sulphate.

There are various advantages of using anammox bacteria in nitrogenous wastewater treatment compared to conventional nitrification-denitrification method. Leachate contains high ammonium concentrations and low biodegradable organic material ratio (Kimura *et al*, 2009). Hence, additional carbon sources need to be added in order for

ammonium breakdown by nitrifying and denitrifying bacteria. However, anammox bacteria do not require additional carbon sources for ammonium oxidation. Besides, anammox bacteria produce less sludge from anaerobic ammonium oxidation process compared to nitrifying and denitrifying bacteria (Fux *et al.* 2010). Anammox bacteria oxidizes ammonia to nitrogen gas under anoxic condition, hence no additional oxygen is required compared to aerobic nitrifying and denitrifying bacteria (Kimura *et al.*, 2009).

The objective of this research is to characterize the anammox bacteria from leachate sample collected from local wastewater treatment plant. As anammox bacteria present a good prospect for effective nitrogen removal from nitrogenous wastewater, characterization of anammox bacteria were essential to improve the quality of discharged effluent in wastewater treatment plant and enhance the knowledge about anammox bacteria in local system.

Characterization of anammox bacteria involves cultivation and enrichment, physiological analysis, morphological analysis, and molecular analysis. Anammox bacteria were cultivated in an anaerobe jar using anammox selective medium. Physiological analysis of the bacteria involves quantification of nitrate, nitrite and ammoniacal nitrogen from anammox metabolism. This analysis is necessary to identify presence of anammox activity in anammox enrichment culture. Anammox bacteria present in enrichment culture and leachate sample were identified using molecular analysis, nucleic acid extraction and PCR.

2.0 LITERATURE REVIEW

2.1 Anammox Bacteria in Wastewater Treatment Plants

Biological nitrogen removal by bacteria plays an important role in wastewater treatment. Conventionally, it involves nitrification and denitrification process to remove nitrogen from ammonium-rich leachate (Egli *et al.* 2001). The existence of such bacterium was traced around in 1965, but only studied since the past 20 years (Meyer *et al.* 2005). Consequent studies of anammox bacteria reveal the presence of significant populations of anammox bacteria in various wastewater and freshwater ecosystem (Jetten *et al.* 2005).

Anammox bacteria was firstly identified and studied in a fluidized bed-reactor in Delft, Netherlands in early 1990s (Mulder *et al.* 1995). Ammonium loss with an increase in nitrogen gas production under anaerobic condition was observed in the reactor during experiments on denitrifying pilot plant of a multi-stage wastewater treatment system. The process was named as anaerobic ammonium oxidation (anammox) process (Jetten *et al.* 1999). Subsequent studies of anammox bacteria in the following years identified the bacteria as 'missing lithotroph from nature' from phylum *Planctomycetales* (Strous *et al.* 1999). Anammox bacteria were discovered as a new deep branching member of *Planctomycetales*. Currently, five genera of anammox bacteria have been identified; *Candidatus Brocadia*, *Candidatus Kuenenia*, *Candidatus Scalindua*, *Candidatus Anammoxoglobus*, and *Candidatus Jetten* (Ran Li *et al.* 2009).

Anammox bacteria have potential in wastewater treatment and advantages over conventional method. Treatment of nitrogenous wastewater is basically accomplished

through conventional nitrification and denitrification methods (Egli *et al.*, 2001). However, nitrifying and denitrifying bacteria usually require carbon sources and oxygen supply. This is because several types of wastewater have high concentrations of ammonium and low concentrations of biodegradable organic compound (low C/N ratio). For example, wastewater from the dewatering of digested sludge and landfill leachate (Kimura *et al.*, 2009) where additional carbon sources and oxygen must be supplied to nitrifying and denitrifying bacteria in order for effective nitrogen removal. In addition, electric-gobbling machines are needed to aerate the sludge for effective nitrification and denitrification process to take place (Kimura *et al.*, 2009). This will lead to an increase in operational costs. Therefore, anammox bacteria is considered as a promising nitrogen removal method compared to nitrification and denitrification as it does not require additional carbon source, works under anoxic condition and quite efficient in removing nitrogen. Anammox process also produces twice the amount of nitrogen gas per mol of nitrite consumed and increases production of nitrogen gas in sediments where nitrification is limited (Penton, 2009). Since it is anaerobic bacteria, oxygen additions can be reduced up to 60% and finally, the bacteria only produces very little biomass (Fux *et al.*, 2010).

2.2 Properties of Anammox Bacteria

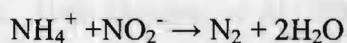
The anammox bacteria have a slow growth rate with a doubling time estimated as 11 days (Strous. *et al.*, 1999). The yield of the anammox bacteria have been determined to be 0.066 C mol biomass mol⁻¹47 ammonium consumed, and the maximum ammonium consumption rate is ~45 mol mg⁻¹ protein min. This shows that anammox bacteria are efficient organism for oxidation of ammonium. Anammox bacteria are reversibly inhibited by very low levels of oxygen, approximately <1μM. Besides, it is also irreversibly

inhibited by very high nitrite concentrations, >10mM (Strous et al. 1997, 1999a, 2002). Anammox bacteria possess a special membrane bound organelle called anammoxosome. Anammoxosome acts as the respiration center in anammox bacteria, as the mitochondria in Eukaryotes (van Niftrik *et al.*, 2004). This organelle is surrounded by a bilayer membrane called ladderane lipids. This membrane protects the anammox bacteria from its toxic metabolic intermediate, hydrazine. ladderane lipids are made from pentacycloanammoxicacids, which contain five linearly concatenated cyclobutane rings (Mascitti *et al.*, 2004).

Methanol and ethanol also act as anammox inhibitors. At concentrations of methanol and ethanol below 1mM, anammox bacteria are severely inhibited. Basically, anammox bacteria use ammonium for anammox reaction. But, recent discoveries show that it is also able to use propionate and acetate as energy source for the reduction of nitrite and nitrate (Hao *et al.* 2004). Anammox bacteria are also identified to conduct carbon fixation pathways but, it is still unknown. Studies conducted on ^{13}C , indicates that reductive pentose phosphate the reductive acetyl-CoA pathway might be the possible carbon dioxide fixation pathway by anammox bacteria (Schmidt *et al.*, 2003).

2.3 Physiology and Biochemistry of Anammox Bacteria

Anammox process is a chemolithotrophic process where energy is obtained from oxidation of inorganic compounds (Galan *et al.*, 2008). In this reaction, 1 mol of ammonium is oxidized by 1 mol of nitrite to produce N_2 gas in the absence of oxygen (Van de Star, 2008),



Hydroxylamine and hydrazine are intermediates in the anammox reaction (Strous *et al.*, 1999). The enzyme responsible in anammox reaction is hydrazine oxidoreductase which is stored in ladderane lipids in anammoxosome. Anammoxosome is a unique and distinct prokaryotic organelle which houses the anammox process. Anammox bacteria are slow growing bacteria depending on nitrite source. In laboratory, the doubling time of anammox bacteria consumes 11 days under optimum (Jetten *et al.*, 2005).

Several parameters which are important in anammox process are concentration of ammonium, nitrite, nitrate and nitrogen gas. Ammonium is oxidized with nitrite in the absence of oxygen to form nitrogen and nitrate. There was no reaction observed when only ammonium or nitrite was added. Furthermore, production of nitrogen gas increases linearly with increasing ammonium usage while concentration of nitrite was constant (Egli *et al.*, 2001). However, anammox reaction was inhibited at high nitrite concentrations due to nitrite toxicity to several bacteria at high concentration (Kimura *et al.*, 2010). For example, growth of pathogenic bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are inhibited by high nitrite concentrations (Schlag *et al.*, 2007).

2.4 Application of Anammox Bacteria

Application of anammox bacteria in nitrogen removal process mainly revolves around wastewater and effluent treatment process. Use of anammox bacteria for nitrogen removal process will consequently reduce costs for aeration and oxygen supply in wastewater treatment process. (van Dongen *et al.*, 2001). Anammox bacteria are also being researched for application in wastewater treatment plant in combination with partial

nitrification-anammox nitrogen removal method. For example, CANON (Completely Autotrophic Nitrogen removal Over Nitrite), SHARON (Single reactor for High activity Ammonia Removal Over Nitrite) and OLAND (oxygen-limited autotrophic nitrification-denitrification) process. SHARON and OLAND process involving combination of partial nitrification and anammox process for nitrogen removal while CANON-anammox involves conversion of half of the ammonium present in wastewater to nitrite to be utilized by anammox bacteria later (Li *et al.*, 2008).

Apart from application in wastewater treatment, anammox bacteria are also a promising benchmark for hydrazine production in future. Hydrazine is the intermediate metabolic in anammox reaction (Schmidt *et al.*, 2003). Insight into the genes and proteins involved in this reaction may enable expression and further optimization of the production of this potent fuel in a suitable biological system. Hydrazine is mainly used high-energy rocket fuel. Furthermore, it is also used as a reactant in military fuel cells, nickel plating, polymerization of urethane, for removal of halogens from wastewater, as an oxygen scavenger in boiler feed water to inhibit corrosion, and in photographic development (Von Burg *et al.*, 1991).

3.0 MATERIALS AND METHOD

3.1 Sample collection

Leachate sample was obtained from local wastewater treatment plant for cultivation of anammox bacteria. Sample collected consist of nitrogenous leachate and biomass for treatment in anoxic tank. It was brownish in color with dark brown biomass. The sample was collected from anoxic tanks from the waste water treatment plant since anammox bacterium grows in anoxic conditions.

3.2 Cultivation and Enrichment of Anammox Bacteria

3.2.1 Anammox Selective Medium

Culturing of anammox bacteria were carried out using sample inoculated with medium containing 21 trace elements (Egli *et al.*, 2001). Anammox bacteria were enriched in 250ml Schott flasks containing approximately 21 mineral medium with ammonium as electron donor, nitrite as electron acceptor and bicarbonate as carbon source. The bottles were incubated in GasPak anaerobic jar. The initial pH was 7 and adjusted with HCl every two weeks. pH of the enrichment culture was tested using pH meter and adjusted with HCl solution. The medium contained initially in demineralized water: 3 mM $(\text{NH}_4)_2\text{SO}_4$, 6 mM NaNO_2 , 25 mM KHCO_3 , 1 mM K_2HPO_4 , 0.5 mM CaCl_2 , 0.5 mM MgCl_2 , 2 ml of trace element solution 1 and 1 ml trace element solution 2 per liter. Trace element solution 1 contained (per liter demineralized water) 10 g $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$ and 5 g FeSO_4 . Trace element solution 2 contained (per liter demineralized water) 15 g $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$, 0.43 g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 0.24 g $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 0.99 g $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$, 0.25 g

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.22 g $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.19 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.08 g Na_2SeO_3 , 0.014 g H_3BO_4 .

3.2.2 Nutrient and Anammox Selective Agar

Culturing of anammox bacteria in agar plates were also attempted in two types of agar, firstly, in nutrient agar and secondly, in agar containing anammox selective medium. Four agar plates containing anammox selective medium were used in culture of anammox bacteria. The agar plates were prepared by mixing 6g of agar powder with 125ml anammox selective medium. Secondly, 6 agar plates were prepared by mixing 125ml distilled water with 6g of nutrient agar. The agar plates were then streaked by using leachate sample which contains bacteria. The leachate sample was used with 0 and 10^{-5} dilution. Two types of agar were used to culture the anammox bacteria to compare the anammox bacterial growth in different types of agar, analyze the nutrient needs of anammox bacterium and isolate the bacterial culture. The agar plates were placed in gas tight GasPak anaerobic jar at 37°C and incubated for two weeks .

3.3 Morphological analysis

Gram staining technique was applied to distinguish the bacteria, whether it is Gram positive or Gram negative bacteria. Suspension from anammox selective medium enrichment culture was used to detect the presence of bacterial cells in enrichment culture. The technique was conducted using standard Gram Staining method (Pommerville, 2001).

3.4 Physico-chemical Analysis of Anammox Bacteria

To determine whether the anammox bacterial culture performed the anammox reaction, metabolites involved in anammox reaction, ammoniacal-nitrogen, nitrate and nitrite were tested (Egli *et al.*, 2001). Hence, ammoniacal nitrogen, nitrate, and nitrite concentrations were measured spectrophotometrically according to Standard Methods for Water and Wastewater Examination (1998) using Spectrophotometer HACH DR2800-1. Concentration of nitrate was measured by using cadmium reduction method. Ten μL of sample were mixed with NitraVer 3 Nitrite Reagent Powder Pillow (100/pkg) and measured at 507nm. Concentration of nitrate was measured by using cadmium reduction method. Ten μL of sample were used with NitraVer 3 Nitrite Reagent Powder Pillow (100/pkg) at 507 nm. Concentration of ammoniacal nitrogen was measured by using salicylic method. Ten μL of sample were mixed with Ammonia Salicylate Reagent Powder Pillow (100/pkg) and measured at 655nm. Then, after 3 minute reaction, Ammonia Cyanurate Reagent Powder Pillow (100/pkg) were added and after 15 minute reaction, concentration of ammoniacal nitrogen in sample were measured.

3.5 DNA Extraction

Genomic DNA was extracted from anammox bacteria from two sources, enrichment culture and directly from leachate sample. The extraction was carried out with slight adaptation from Schmidt *et al* (1991) where the supernatant of the leachate sample were concentrated prior to DNA extraction. Supernatant of the enrichment culture and leachate sample was concentrated three folds and centrifuged 2 minutes on 14,000rpm using table top centrifuge. The bacterial cell pellet was resuspended in 567 μL of TE buffer, 3 μL of 20mg/ml Proteinase K, and 30 μL sodium dodecyl sulfate (SDS) and incubated for 1 h at

37°C. Next, 100µl of 5M NaCl and 80µl of NaCl/CTAB solution were added. The mixture were mixed and incubated for 10 minutes at 65°C. The protein and polysaccharide complexes were removed by extraction with an equal volume of chloroform-Isoamyl alcohol (24:1), followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated by the addition of 0.6 volumes of isopropanol and centrifugation for 2 min at 13,000 rpm. The pellet was suspended in 1×TE buffer. Genomic DNA was analyzed using 1% Agarose Gel Electrophoresis. Fifty ml of 1×TAE buffer were mixed 0.5g of agarose powder and molded into gel tray with comb. Five µL of sample were mixed with 1µl of 6x loading dye and loaded into wells. Electrophoresis was performed at 80V for 45 minutes. After electrophoresis, the gel were stained with 1% ethidium bromide solution for 3 minutes and destained for 10 minutes before viewing under Alpha DigiDoc RT UV transilluminator.

3.6 DNA Quantification

The absorbance at 260nm (A260) was used to determine the quantity of DNA. The ratio of A260 to A280 values indicated the relative purity of the DNA. Five µl of DNA sample were mixed with 995µl of sterile distilled water and analyzed using Amersham Pharmacia Ultraspec 1100p UV Spectrophotometer. DNA extracts were stored at -20°C for further uses.

3.7 16S rRNA Gene Amplification

16S rRNA gene in anammox bacteria were amplified according to Ran-Li *et al.* (2009) using the *Planctomycetes* 16S rRNA specific gene primer as shown in table 3.7.

Table 3.7: 16S rRNA *Planctomycetes* specific gene primer sequence and annealing temperature

Target	Primer and Sequence	Ta (°C) ^a
<i>Planctomycetes</i> 16S rRNA gene	Pla46f: 5'-GGATTAGGCATGCAAGTC-3' 1390r: 5'-ACGGGCGGTGTGTACAA-3'	59

In all PCR amplifications, reactions were performed with Taq DNA Polymerase with a total volume of 25 µL and 2µl of DNA added as template. All PCR program consisted of an initial 5min denaturation at 95 °C, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing for 1 min, extension at 72 °C for 1.5 min, and a final elongation step of 10 min at 72 °C. PCR products were analyzed on a 1% (w/v) agarose gel and purified by using Fermentas Gel Extraction Kit. PCR products were analyzed using 1% Agarose Gel Electrophoresis. Fifty ml of 1x TAE buffer were mixed with 0.5g of agarose powder and molded into gel tray with comb. 5µl of sample were mixed with 1µl of 6x loading dye and loaded into wells. Electrophoresis was performed at 80V for 45 minutes. After electrophoresis, the gel was stained with 1% ethidium bromide for 3 minutes and destained for 10 minutes before viewing under Alpha DigiDoc RT UV transilluminator.

3.7.1 PCR Optimization

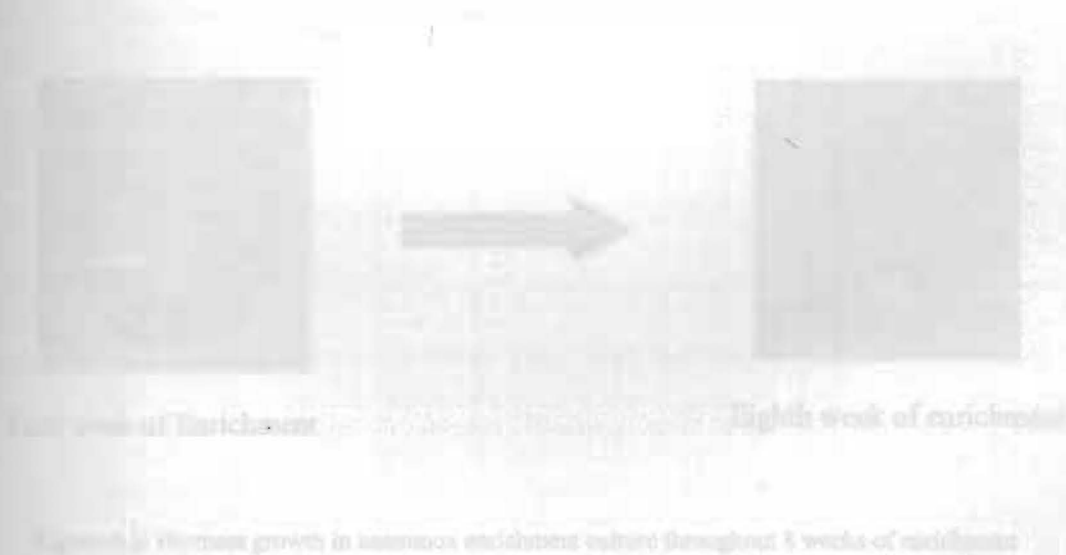
PCR amplification of anammox bacteria were optimized by two methods, firstly, by increasing number of cycles and secondly, gradient PCR. Number of cycles for PCR was increased accordingly, 30, 35, 40, 45 and 50 and this was performed in Sensoquest Labcycler. Other parameters such as reagent concentration, volume, and temperature profile were kept constant. Range of annealing temperature used in gradient PCR is 55°C-65°C and this was performed in Sensoquest Labcycler. Other parameters such as reagent concentration, volume and number of cycles in PCR were kept constant.

3.7.2 PCR Product Purification 4.0 RESULTS

PCR products were purified by gel extraction method using Fermentas DNA Purification Kit. 50µl of PCR product were loaded into gel with 10µl of 6X loading dye. Electrophoresis was performed at 80V for 45 minutes.

3.8 Sequencing and Analysis

The purified PCR products were sent to a commercial company First Base Laboratory Sdn. Bhd, Malaysia for direct sequencing. Sequencing results were blasted using BLAST in NCBI Database to find similar sequences.



4.0 RESULTS

4.1 Cultivation and Enrichment of Anammox Bacteria

4.1.1 Enrichment of anammox bacteria in culture

Cultivation of anammox bacterium was carried out using anammox selective medium. On the first day of culture, only small amount biomass of the leachate was observed in enrichment culture. Gradually, the amount of the biomass increased throughout the eight week of enrichment in the culture. Growing and agglomeration of the biomass in culture indicated presence of microorganism. Biomass in enrichment culture was found to start agglomerate two weeks after culturing. As the biomass grow, the color of the biomass in enrichment culture turned more brownish towards the end of the eighth week.



First week of Enrichment



Eighth week of enrichment

Figure 4.1: Biomass growth in anammox enrichment culture throughout 8 weeks of enrichment